Year : 2013

Bases moléculaires de la dysplasie pseudorhumatoïde progressive

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UNIVERSITE DE LAUSANNE - FACULTE DE BIOLOGIE ET DE MEDICINE

Département de Pédiatrie
Service de Pédiatrie Moléculaire

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THESE

préparée sous la direction du Docteur Luisa Bonafé
et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

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Médecin diplômée de l'Espagne
Originaire de Valencia (Espagne)

Lausanne
2013

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale
Doctorat en médecine

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Directeur de thèse	*Madame le Docteur* ***Luisa Bonafé***
Co-Directeur de thèse	
Expert	*Madame le Professeur* ***Martine Jotterand***
Directrice de l'Ecole doctorale	*Madame le Professeur* ***Stephanie Clarke***

la Commission MD de l'Ecole doctorale autorise l'impression de la thèse de

madame Nuria Garcia depraz

intitulée

Bases moléculaires de la dysplasie pseudorhumatoïde progressive

Lausanne, le 18 juin 2013

pour Le Doyen
de la Faculté de Biologie et de Médecine

Madame le Professeur Stephanie Clarke
Directrice de l'Ecole doctorale

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La dysplasie pseudorhumatoïde progressive (PPRD) est une arthropathie non-inflammatoire causée par des mutations récessives du gène WISP3. Elle se manifeste pendant la petite enfance avec une raideur progressive des articulations et des douleurs. Les patients sont référées habituellement aux rhumatologues pédiatres et aux orthopédistes, et seulement dans un deuxième temps, vers les généticiens et/ou experts dans les dysplasies osseuses. Pour cette raison le diagnostic clinique, qui repose sur les signes radiologiques typiques et l'expérience clinique, est souvent retardé et les patients peuvent recevoir des traitements anti-inflammatoires et immunosuppresseurs innécessaires.

Nous reportons ici une large série de patients atteints de PPRD avec confirmation du diagnostic au niveau moléculaire, et nous soulignons les caractéristiques cliniques et radiologiques de la maladie.

Il existe une fenêtre d'âge dans laquelle les signes radiologiques sont spécifiquement reconnaissables. Des anomalies spondyloépiphyseales très légères peuvent apparaître avant l'âge de 9 ans et une perte de cartilage non-spécifique qui ressemble à une ostéoarthrite avancée est présente chez les jeunes adultes. Les articulations interphalangiennes sont les premières à être atteintes, suivis par les genoux et les hanches. L'atteint de la colonne arrive chez les enfants plus grands et dans l'adolescence. Une cyphose est fréquente et une scoliose survienne chez une minorité de patients. Des signes d'ostéoarthrite avancée comme des formations ostéophytiques et/ou des calcifications périarticulaires sont observés chez les jeunes adultes atteints de PPRD et peuvent être responsables d'une certaine inflammation secondaire.

L'analyse moléculaire du gène WISP3 par séquençage de l'ADN génomique permet de confirmer le diagnostic dans la plupart des cas. Néanmoins, des splicings alternatives causés par des mutations introniques peuvent être détectés dans le cDNA des fibroblastes. Dans le cas où l'analyse génomique ne montre aucune mutation chez un individu présentant les signes et symptômes typiques de la maladie, une biopsie de peau est indiquée pour analyse moléculaire du cDNA.

La prise en charge de la PPRD est symptomatique et largement insatisfaisante. Le remplacement des articulations les plus atteintes est souvent nécessaire dès l'adolescence afin de diminuer les douleurs et maintenir la mobilité.

American Journal of Medical Genetics Part C (Seminars in Medical Genetics) 160C:217–229 (2012)

ARTICLE

The Diagnostic Challenge of Progressive Pseudorheumatoid Dysplasia (PPRD): A Review of Clinical Features, Radiographic Features, and *WISP3* Mutations in 63 Affected Individuals

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Progressive pseudorheumatoid dysplasia (PPRD) is a genetic, non-inflammatory arthropathy caused by recessive loss of function mutations in *WISP3* (Wnt1-inducible signaling pathway protein 3; MIM 603400), encoding for a signaling protein. The disease is clinically silent at birth and in infancy. It manifests between the age of 3 and 6 years with joint pain and progressive joint stiffness. Affected children are referred to pediatric rheumatologists and orthopedic surgeons; however, signs of inflammation are absent and anti-inflammatory treatment is of little help. Bony enlargement at the interphalangeal joints progresses leading to camptodactyly. Spine involvement develops in late childhood and adolescence leading to short trunk with thoracolumbar kyphosis. Adult height is usually below the 3rd percentile. Radiographic signs are relatively mild. Platyspondyly develops in late childhood and can be the first clue to the diagnosis. Enlargement of the phalangeal metaphyses develops subtly and is usually recognizable by 10 years. The femoral heads are large and the acetabulum forms a distinct "lip" overriding

Nuria Garcia Segarra and Laureane Mittaz contributed equally to this work.
Grant sponsor: Swiss National Research Foundation; Grant number: 310030_132940.
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DOI 10.1002/ajmg.c.31333
Article first published online 12 July 2012 in Wiley Online Library (wileyonlinelibrary.com).

the femoral head. There is a progressive narrowing of all articular spaces as articular cartilage is lost. Medical management of PPRD remains symptomatic and relies on pain medication. Hip joint replacement surgery in early adulthood is effective in reducing pain and maintaining mobility and can be recommended. Subsequent knee joint replacement is a further option. Mutation analysis of *WISP3* allowed the confirmation of the diagnosis in 63 out of 64 typical cases in our series. Intronic mutations in *WISP3* leading to splicing aberrations can be detected only in cDNA from fibroblasts and therefore a skin biopsy is indicated when genomic analysis fails to reveal mutations in individuals with otherwise typical signs and symptoms. In spite of the first symptoms appearing in early childhood, the diagnosis of PPRD is most often made only in the second decade and affected children often receive unnecessary anti-inflammatory and immunosuppressive treatments. Increasing awareness of PPRD appears to be essential to allow for a timely diagnosis.

KEY WORDS: progressive pseudorheumatoid dysplasia; *WISP3*; joint pain

How to cite this article: Garcia Segarra N, Mittaz L, Campos-Xavier AB, Bartels CF, Tuysuz B, Alanay Y, Cimaz R, Cormier-Daire V, Di Rocco M, Duba H-C, Elcioglu NH, Forzano F, Hospach T, Kilic E, Kuemmerle-Deschner JB, Mortier G, Mrusek S, Nampoothiri S, Obersztyn E, Pauli RM, Selicorni A, Tenconi R, Unger S, Utine GE, Wright M, Zabel B, Warman ML, Superti-Furga A, Bonafé L. 2012. The diagnostic challenge of progressive pseudorheumatoid dysplasia (PPRD): A review of clinical features, radiographic features, and WISP3 mutations in 63 affected individuals. Am J Med Genet Part C Semin Med Genet 160C:217–229.

INTRODUCTION

Progressive pseudorheumatoid dysplasia (PPRD) (MIM 208230) is an autosomal recessive inherited skeletal dysplasia characterized by progressive non-inflammatory arthropathy affecting primarily the articular cartilage. Since its initial description by Wynne-Davies et al. [1982] and independently by Spranger et al. [1983a,b] several cases or small series have been published, mostly reporting clinical and radiological features [Kaibara et al., 1983; Al-Awadi et al., 1984; Legius et al., 1993; el-Shanti et al., 1997; Cogulu et al., 1999; Mampaey et al., 2000]. PPRD has an estimated incidence of 1 per million in the UK [Wynne-Davies et al., 1982] and seems to be more frequent in the Middle East and Gulf states [Teebi and Al Awadi, 1986] as well as in Turkey, where a founder effect has been suggested [Delague et al., 2005].

Initially mapped to chromosome 6q22 [el-Shanti et al., 1998; Fischer et al., 1998], the disorder has been subsequently associated with mutations in *WISP3* (Wnt1-inducible signaling pathway protein 3; MIM 603400), a member of the *CCN* (CTGF, cyr61/cef10, nov) gene family [Hurvitz et al., 1999]. CCN proteins are growth factors that regulate cell proliferation, differentiation, migration, and adhesion in connective tissue [Yang and Lau, 1991; Kireeva et al., 1997; Lau and Lam, 1999]. *WISP3* encodes a 354 amino acid protein including, as other WISP proteins, specific structural domains (http://www.uniprot.ch/; Uniprot number: O95389): an insulin-like growth factor binding protein (IGFBP) domain, probably responsible for IGF-1 signaling modulation in the growth plate [Sen et al., 2004; Cui et al., 2007; Yang and Liao, 2007], a thrombospondin type I (THBS)-like domain, and a cysteine knot domain. *WISP3* has a low level of expression [Hurvitz et al., 1999]; its transcript has been detected mainly in mesenchymal cells and tissues [Pennica et al., 1998; Hurvitz et al., 1999] including chondrocytes, synoviocytes, and bone marrow progenitor cells induced to undergo chondrogenesis in vitro [Hurvitz et al., 1999]. The biological function of WISP3 has been studied ex vivo: it plays a role in controlling expression of collagen II, aggrecan, and SOX9 in chondrocyte cell lines [Sen et al., 2004]; it regulates superoxide dismutase activity and its absence correlates with increased reactive oxygen species [Miller and Sen, 2007]; human chondrocytes from a single PPRD patient showed increased cell proliferation and abnormal matrix metalloproteinase processing [Zhou et al., 2007] as suggested also by studies on osteoarthritic chondrocytes [Baker et al., 2012]. Since WISP3 depletion or overexpression causes no pathological phenotype in mice [Kutz et al., 2005; Nakamura et al., 2009], in vivo studies were performed in zebrafish, showing that overexpressed zWISP3 antagonizes BMP and Wnt signaling, whereas depletion of zWISP3 using mutants causing PPRD in humans had no or reduced inhibitory activity on this signaling [Nakamura et al., 2007]. These data suggest that dysregulation of BMP and/or Wnt signaling may contribute to cartilage degeneration in humans with PPRD. However, the exact pathomechanism in human PPRD cartilage is still unclear and a specific treatment not yet available. The aim of this study was to report on the main clinical features of patients referred to our center and to describe the mutation spectrum of *WISP3* in this group of patients.

MATERIALS AND METHODS

Patients

Eighty-one patients from 66 families were referred to us for mutation analysis of the *WISP3* gene because of a diagnostic suspicion of PPRD. Prior to mutation analysis, a clinical and radiographic documentation was requested. The clinical and radiological phenotype of each patient was reviewed by the expert clinicians in our team and classified as either typical, or atypical but possibly compatible with PPRD. Patients with features that were clearly not compatible with PPRD were excluded from analysis. Patients with the designation of typical PPRD fulfilled the following clinical criteria: onset in early childhood, stiffness and pain of multiple joints, enlargement

of the interphalangeal joints (IPJ), normal inflammatory parameters, and absence of extra-skeletal manifestations. Radiological criteria for PPRD were: metaphyseal enlargement of IPJ, reduced articular space with large dysplastic epiphyses at hips and knee, platyspondyly with anterior beaking of vertebral bodies, absence of articular bone erosion, and generalized osteopenia starting in adolescence. Among the 81 patients sent with the diagnostic suspicion of PPRD, 64 (from 49 families) fulfilled the clinical–radiological criteria for typical PPRD.

Patients with atypical forms presented seronegative arthropathies with either very early (before 3 years) or late (after 8 years) age of onset, or with absence of IPJ and/or spine involvement. Patients with additional extra-skeletal manifestations or with painless joint stiffness/contractures were also considered atypical.

Molecular Study

EDTA blood samples or extracted DNA were obtained from all patients (except for Patient 25 for whom only parental DNA was supplied); parental blood/DNA was also obtained and analyzed, when available (in 25 out of 49 families). Skin biopsies were collected from Patients 27, 48, 49, and 50. Appropriate informed consent for genetic testing was obtained from all individuals. Molecular testing was performed in the context of a study approved by the Ethic Committee of the University of Lausanne as well as diagnostic service.

WISP3 Genomic Sequencing

Genomic DNA was extracted from blood leukocytes according to standard protocols. The five coding exons of *WISP3* (GenBank accession no *NM_003880*; Ensembl ENST00000368666), the intron–exon boundaries, as well as the additional *WISP3* exon (exon 3b), located between exons 3 and 4 and alternatively spliced after exon 3 (Ensembl ENST00000368664) were amplified from genomic DNA by PCR and directly sequenced. Primers and conditions are available upon request. Each variant was confirmed in a second amplification product. Genomic DNA of 50 control individuals of white ethnicity and various origins (German, Italian, British, French, Swiss, Australian, Canadian) was studied for all novel putative *WISP3* mutations (Table I).

WISP3 cDNA analysis

In order to identify the second mutation in two patients (Patients 48 and 49) with only 1 heterozygous *WISP3* mutation detected at genomic level and to search for mutations in the lone typical PPRD patient with no discernible mutation (Patient 50), we investigated the WISP3 mRNA in cultured skin fibroblasts obtained from these patients.

Skin fibroblasts were cultured in Dulbecco's modified Eagle's Medium with 10% fetal calf serum and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml), at 37°C with 5% CO_2. Total RNA was extracted from confluent fibroblasts using TRIZOL Reagent (Invitrogen Life Technologies, Carlsbad, CA) and was kept at −80°C. When needed, fibroblasts were treated with cycloheximide (28 μg/ml, 4 hr), according to published protocols [Rajavel and Neufeld, 2001] in order to prevent nonsense mediated RNA decay (NMRD). Double stranded cDNA was generated using the primescript RT-PCR kit (Takara Bio Inc., Otsu, Japan).

The entire *WISP3* cDNA was amplified by PCR using oligonucleotides 5utrF2 (5′-gctccacggtcccagcgac-3′) and 3utrR1 (5′-tatgacaggattgactaacttttcc-3′); cycling conditions: initial denaturation step (94°C for 5 min), 40 amplification cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min 30 sec), final extension (72°C for 5 min). Nested PCRs were performed on initial PCR products generated on cDNA using internal oligonucleotides (sequences available upon request). Cycling conditions were as above with a shorter extension time (30 sec), except that 35 cycles were performed, the 10 first being "touchdown" cycles with annealing between 65 and 55°C. All RT-PCR amplified fragments were analyzed by fluorescent bidirectional direct sequencing as mentioned above.

RESULTS

From the review of the clinical and radiological data of the 81 patients, 64 patients presented signs and symptoms considered typical of PPRD, whereas 17 had some atypical features. Of these 17 patients, 3 presented with multiple epiphyseal dysplasia with enlarged epiphyses but no spine involvement, 5 had progressive painless contractures, 1 had onset of symptoms only at 15 years, 2 had early onset at 2 years, and 3 had alternative diagnoses (Winchester syndrome due to MMP2 mutations, COL2A1-related disorder, and possible CACP syndrome (coxa vara, arthropathy, camptodactyly, pericarditis)). In three further cases, the radiographic documentation was incomplete. In 63 out of the 64 patients with PPRD, mutations in *WISP3* were identified by genomic and/or cDNA sequencing (Table I). Patients 20 (homozygous for a new mutation) and 39 (heterozygous for the same new mutation) are from the same Polish family. One individual (Patient 50) remains a puzzle, as this patient had been part of one of the original descriptions of PPRD (reported in Spranger et al., 1983b as "Patient 2, B.K."), yet no mutation could be identified despite repeated studies at both genomic and cDNA level. Mutation screening was negative in all 17 atypical patients.

Clinical and Radiographic Features of PPRD Patients With Confirmed WISP3 Mutations

Affected patients were normal at birth and developed normally during the first years of life. The onset of symptoms was in childhood between 3 and 6 years of age. The interval between onset of symptoms and diagnosis varied between 1 and 34 years; in Turkish patients there was an average delay in diagnosis of 6 years, whereas in non-Turkish patients the mean delay was 13 years.

Symptoms consisted of progressive stiffness of all joints, motor weakness,

TABLE I. Mutations Identified in Patients in the Present Study

Index patient			Allele 1		Allele 2	
#	Origin	Affected sibs	Coding sequence	Protein	Coding sequence	Protein
1	MO	1	c.48 + 2dupT	Splicing	c.48 + 2dupT	splicing
2	?		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
3	IT	1	c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
4	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
5	TR	1	c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
6	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
7	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
8	TR	1	c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
9	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
10	TR	1	c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
11	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
12	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
13	TR	1	c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
14	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
15	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
16	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
17	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
18	TR	1	c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
19	TR		c.156C > A	p.Cys52*	c.156C > A	p.Cys52*
20	PL		182G > T	p.Cys61Phe	182G > T	p.Cys61Phe
21	TR	1	c.185delC	p.Pro62Leufs*4	c.185delC	p.Pro62Leufs*4
22	TR		c.185delC	p.Pro62Leufs*4	c.185delC	p.Pro62Leufs*4
23	TR		c.327C > A	p.Tyr109*	c.327C > A	p.Tyr109*
24	TR		c.327C > A	p.Tyr109*	c.327C > A	p.Tyr109*
25	TR		c.327C > A	p.Tyr109*	c.327C > A	p.Tyr109*
26	TR	1	c.342_343delTG	p.Ala115Ilefs*16	c.342_343delTG	p.Ala115Ilefs*16
27	TR		c.346 + 1G > T	p.Tyr109_Met195delins9	c.346 + 1G > T	p.Tyr109_Met195delins9
28	TR	2	c.708dupC	p.Asn237Glnfs*3	c.708dupC	p.Asn237Glnfs*3
29	TR		c.708dupC	p.Asn237Glnfs*3	c.708dupC	p.Asn237Glnfs*3
30	TR		c.727_731delGAGAA	p.Glu243Lysfs*34	c.727_731delGAGAA	p.Glu243Lysfs*34
31	TR	1	c.727_731delGAGAA	p.Glu243Lysfs*34	c.727_731delGAGAA	p.Glu243Lysfs*34
32	TR	1	c.727_731delGAGAA	p.Glu243Lysfs*34	c.727_731delGAGAA	p.Glu243Lysfs*34
33[a]	D		c.739_740delTG	p.Cys247Leufs*31	c.739_740delTG	p.Cys247Leufs*31
34	TR		c.850G > T	p.Gly284*	c.850G > T	p.Gly284*
35	TR		c.857C > G	p.p.Ser286*	c.857C > G	p.p.Ser286*
36	IN		c.1010G > A	p.Cys337Tyr	c.1010G > A	p.Cys337Tyr
37[b]	IT		c.156C > A	p.Cys52*	c.236-237CC > AA	p.Ala79Glu
38	IT		c.156C > A	p.Cys52*	c.670G > A	p.p.Gly224Arg
39	PL		182G > T	p.Cys61Phe	624dupA	p.Cys209Metfs*21
40	USA	1	c.197G > A	p.Ser66Asn	c.621_622delAAinsT	p.Lys207Asnfs*25
41	IT		c.197G > A	p.Ser66Asn	c.725_726delAA	p.Lys242Argfs*36
42[b]	IT		c.434G > A	p.Cys145Tyr	c.993G > A	p.Trp331*
43	IT		c.589G > A	p.Ala197Glyfs*5	c.725_726delAA	p.Lys242Argfs*36
44	USA		c.621_622delAAinsT	p.Lys207Asnfs*25	624dupA	p.Cys209Metfs*21
45	UK		c.677G > T	p.Gly226Val	c.862_863dupAC	p.Gln289Leufs*25
46	IT		c.866dupA	p.Ser290Glufs*13	c.868-869delAG	p.Ser290Leufs*12
47	IT		c.868_869delAG	p.Ser290Leufs*12	c.1004G > A	p.Cys335Tyr
48	B		c.156C > A	p.Cys52*	c.49-763G > T; r.48_49ins49-749_49-618	p.Phe17Asnfs*42

Table I. (*continued*)

Index patient			Allele 1		Allele 2	
#	Origin	Affected sibs	Coding sequence	Protein	Coding sequence	Protein
49	**D**		c.156C > A	p.Cys52*	**c.589 + 27C > G; r.589_590ins589 + 1_589 + 27**	**p.Ala197Glyfs*5**
50[c]	**D**		wt	wt	wt	wt

Origin of patients: MO, Morocco; IT, Italy; TR, Turkey; IN, India; PL, Poland; B, Belgium; D, Germany. Homozygous mutations are shaded in gray. New mutations are indicated in bold.

Homozygosity in Patient 25 was deduced from results of heterozygous mutations identified in parental DNA, as patient's DNA was unavailable.

[a]Patient 33 was reported by Ehl et al. [2004].

[b]Patients 37 and 42 were reported by Hurvitz et al. [1999] (as Patients 2 and 1, respectively).

[c]Patient 50 was reported by Spranger et al. [1983b] (as Patient 2 "B.K.").

gait disturbances, knobby appearance of interphalangeal joints (IPJ), articular pain, and contractures (Fig. 1). The very first symptoms reported were gait anomalies and fatigability in about 50% of patients for whom this information was available. Otherwise, the first sign was IPJ "swelling" in 30% of cases and some degree of knee deformity in about 20% of cases. Pain was reported as the initial symptom in 15% of patients and developed in all patients. Stiffness was

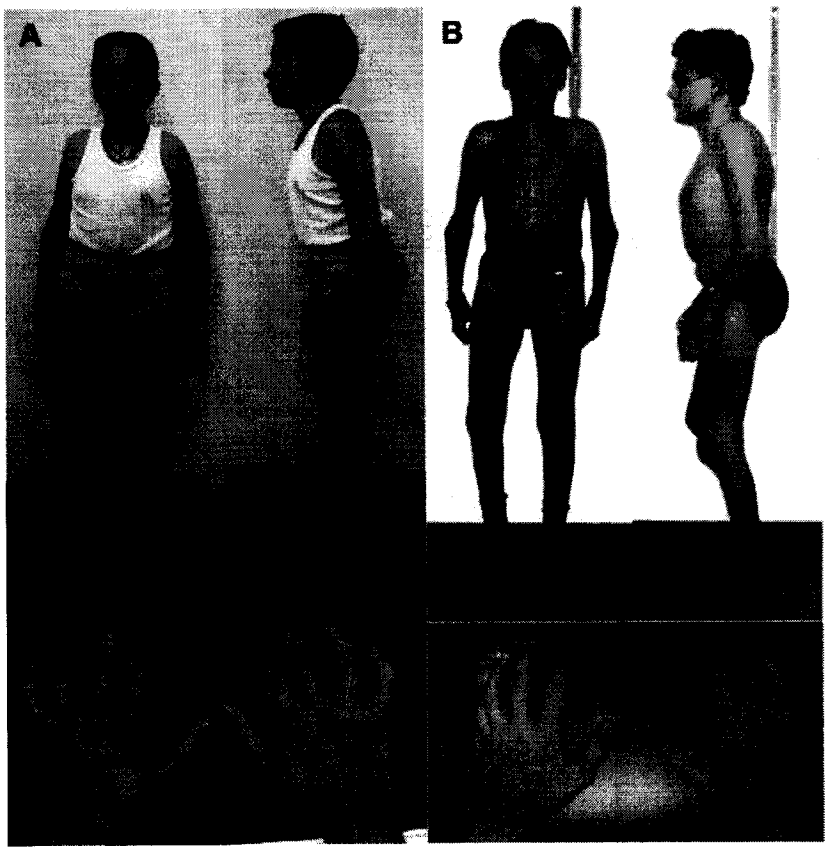


Figure 1. Clinical photographs of two PPRD patients with details of their hands. **A**: Patient 43 (Table I) at age 12 years: note flexum of elbows and knee; there is no trunk/limb disproportion and only initial stiffness of the spine. Camptodactyly and knobby appearance with osseus enlargement of the interphalangeal joints, with no signs of effusion or inflammation. **B**: Patient 42 (Table I) at age 21 years: typical posture of PPRD patients with multiple joint contractures, short trunk and thoracic kyphosis. Marked camptodactyly of all fingers, enlargement of the interphalangeal joints, with no swelling or erythema.

reported initially at IPJ in 60% of patients, followed by knee (45%) and hips (30%); in a minority of patients stiffness was noted at cervical spine, elbow, wrist, and shoulder already at early stages. At last follow-up, all joints (small and large, including spine) were progressively limited in movement. Stature was usually normal at onset of symptoms but was reported as being below percentile 3 at the moment of diagnosis in most cases. Radiological features were spondyloepiphyseal dysplasia with platyspondyly, short and wide femoral neck, large femoral and tibial epiphyses, narrow joint space at hips and knees, enlargement of the epi-metaphyseal portions of metacarpals and phalanges, and osteopenia. Progressive continuous cartilage loss and bone changes similar to osteoarthritis were observed with age. However, the destructive bone erosions typical of juvenile idiopathic arthritis (JIA) were not seen. Figures 2–5 show the main diagnostic radiographic features of molecularly confirmed PPRD patients at different ages. No patients had elevations of inflammatory parameters in blood (erythrocyte sedimentation rate, C-reactive protein) or abnormalities of rheumatological parameters (rheumatic factor, complement, autoantibodies, etc.). There was no improvement with steroids and immune-suppressive drugs (mainly methotrexate and cyclosporine) when these were used.

Ethnic Origin of PPRD Patients

Of the 48 families of patients in whom we identified *WISP3* mutations, 30 are of Turkish origin, 8 are of Italian origin, 7 are of other white ethnicity and non-Mediterranean origin (Germany, Belgium, Poland, UK, and USA), 1 is of Indian origin, 1 is Moroccan, while the ethnic origin of the remaining patient is unknown (see Table I). Of the 30 Turkish families, all affected individuals were found to be homozygous for *WISP3* mutations (16 of them were homozygous for the c.156C > A mutation, 14 were homozygous for other mutations). This finding is likely explained by the high consanguinity rate in the Turkish population. However, given that the c.156C > A mutation accounts for roughly 50% of PPRD alleles in Turkey, the absence of compound heterozygosity is surprising and may indicate that parental consanguinity may be taken into account in the differential diagnosis, biasing our results towards homozygosity by descent.

cDNA Changes in PPRD Patients

Previous expression studies showed that *WISP3* cDNA is well detectable in chondrocytes and synoviocytes but not in fibroblasts [Hurvitz et al., 1999]. However, chondrocytes and synoviocytes are very difficult to obtain for diagnostic purposes and difficult to maintain in culture as primary cell lines. By using special PCR conditions, we optimized amplification of very low levels of constitutional *WISP3* transcript in

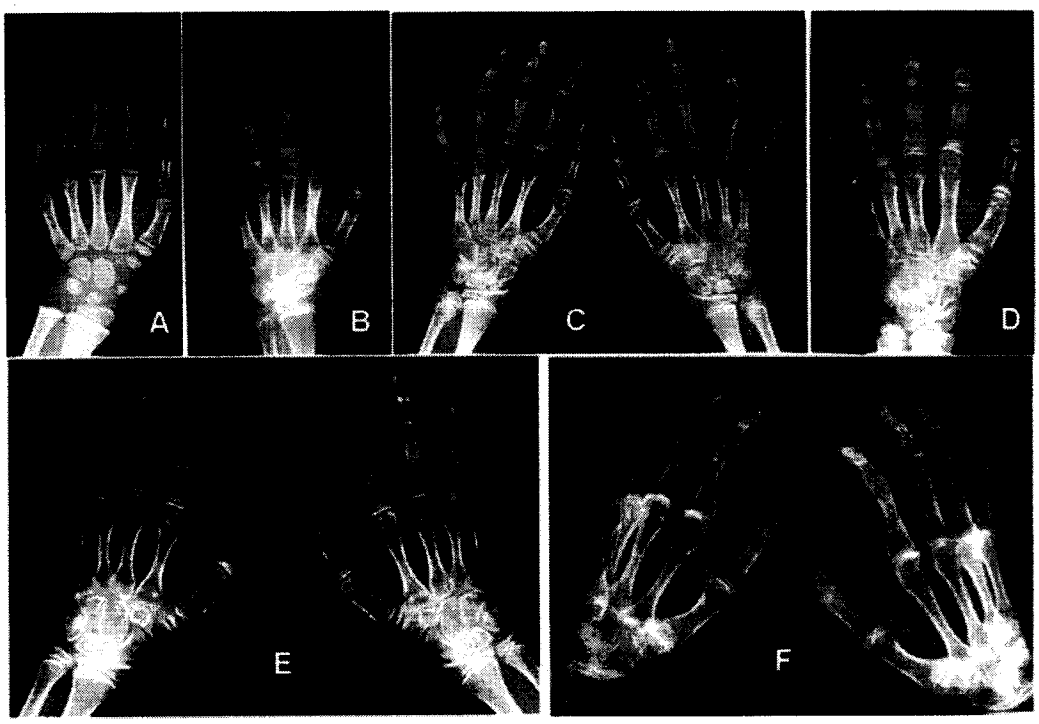


Figure 2. Bone changes in hand X-rays of different PPRD patients at different ages: at 6 years (**A**), minimal metaphyseal enlargement at the interphalangeal joints (IPJ), normal carpal bones; at later ages (**B** = 9 years; **C** and **D** = 10 years; **E** = 12 years) IPJ appear enlarged, with metaphyseal irregularities and the interosseus space between the carpal bones is reduced. Camptodactyly often appears in childhood (C and D = 10 years) and is always present in adulthood (**F** = 45 years) with reduced mobility of all hand joints.

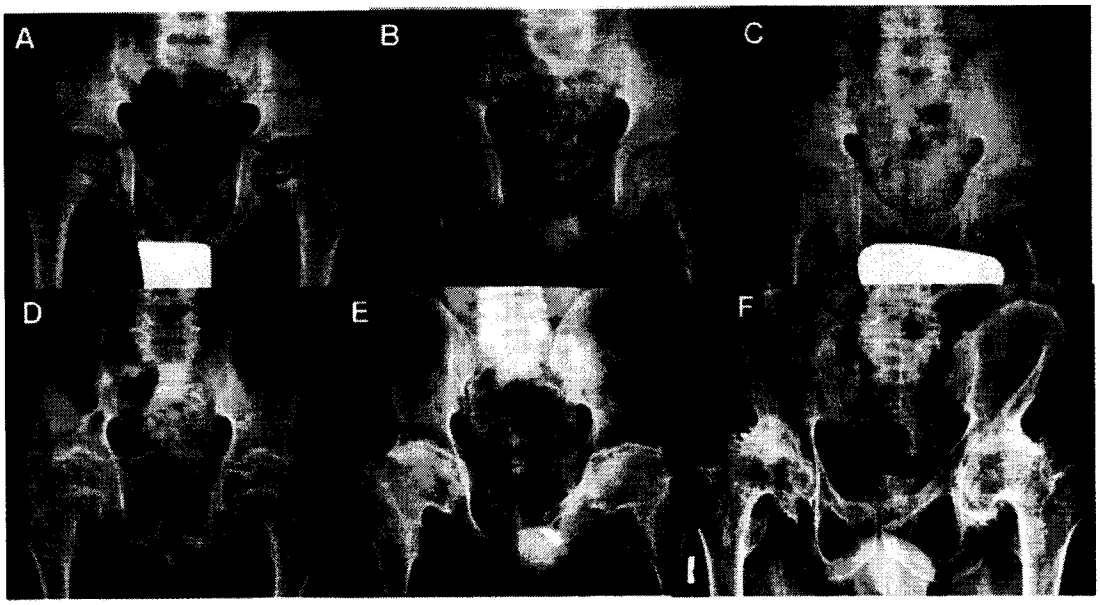


Figure 3. Bone changes in pelvis x-rays in several PPRD patients: at 5 years (**A**), the pelvis appears fairly normal; at later ages (**B** = 6 years, **C** = 10 years, **D** = 12 years) there is reduction of the articular space indicating progressive loss of articular cartilage correlating with progressive hip stiffness and pain. Epiphyses are rather large and become progressively flattened. In adults (**E** = 21 years; **F** = 45 years) there is marked cartilage loss in the interarticular space and osteopenia. The external profile of the acetabula is characteristic, with a distinct "lip" overriding the femuoral neck. D and E are from the same patient at age 12 and 21, respectively.

two control cultured skin fibroblast primary cell lines. In patients with nonsense mutations in both alleles, cycloheximide treatment was used to prevent NMRD. When cycloheximide was tested on control cells, we obtained amplification of two alternative splicing variants, in addition to the wild-type transcript (Fig. 6). The first is a previously reported insertion of the alternatively spliced exon 3b between exons 3 and 4 [Cervello et al., 2004] that leads to a frameshift and introduction of a premature stop codon (p.Ala197Aspfs*24). The second cDNA variant, not previously reported, is a deletion of the last 23 bp of exon 2, due to the use of a potential splice donor site (TAG/gt) 23 bp before the usual splice donor site of exon 2. Again this leads to a frameshift and introduction of a premature stop codon

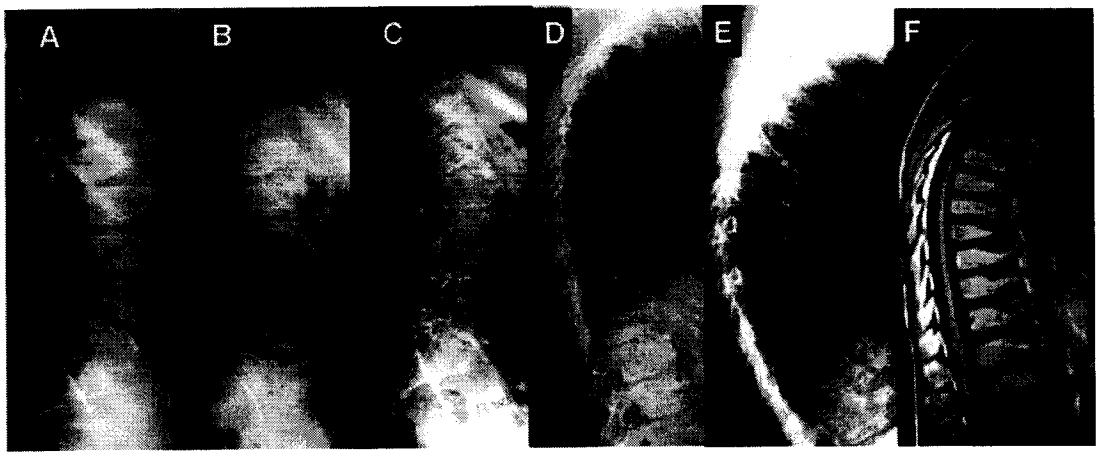


Figure 4. Bone changes in lateral spine X-rays in several PPRD patients at different ages. Involvement of the vertebral bodies may be mild at early stages (**A** = 6 years) and becomes evident with age, with progressive platyspondyly and anterior beaking of the vertebral bodies (**B** = 12 years; **C** = 13 years; **D** = 16 years; **E** = 21 years). Dorsal kyphosis and osteopenia are observed in adolescence (D = 16 years; E = 21 years). **F** = MRI section of lateral thoracic spine at age 12, showing platyspondyly, reduced intervertebral space and anterior beaking of the vertebral bodies.

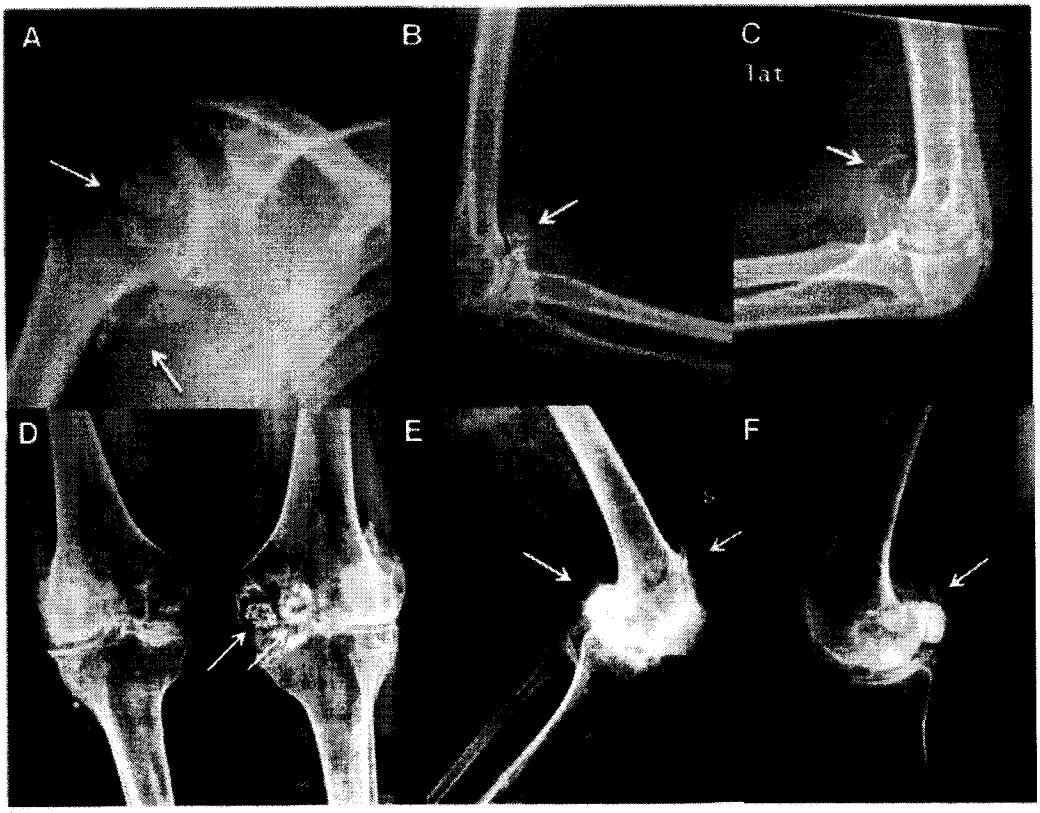


Figure 5. Osteophytic formations (calcified tissues connected to bone) and periarticular calcifications (calcified tissues probably not connected with bone) observed in PPRD patients are indicated with arrows: **A** (shoulder), **D** (knees), **E** (lateral view of left knee), **F** (lateral view of right knee) are from the same 45 year-old patient. **B**: Elbow of a 24-year-old patient. **C**: Elbow of a 16-year-old patient.

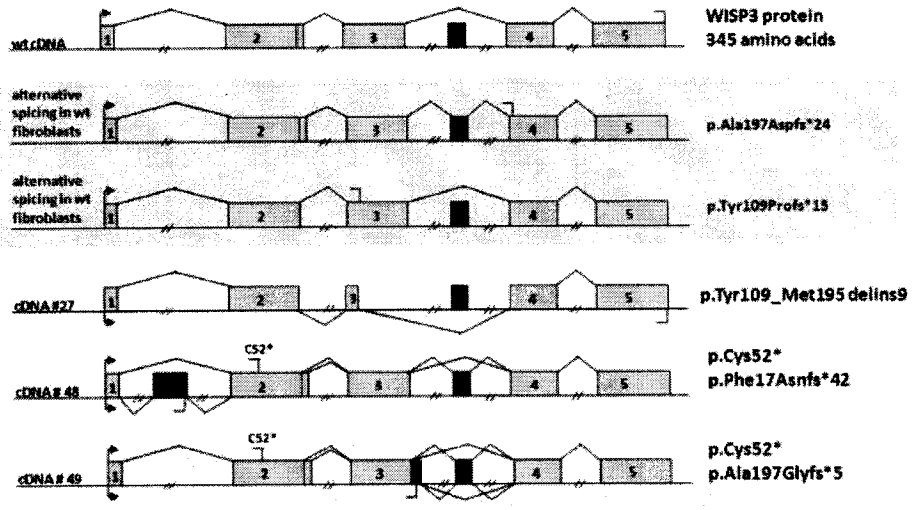


Figure 6. Schematic representation of the different mRNA splice variants detected in control fibroblasts and in fibroblasts from Patients 27, 48, and 49. Exons are presented as numbered boxes, introns as lines. The double slash (//) indicates that the distance is not to scale. Splicing events are indicated by lines above (wt) or below (mutant) the exons. Wild-type coding exons are presented in light gray, the alternatively spliced exon 3b is presented in dark gray, the part of exon 2 alternatively spliced out is separated by a dashed line. Pseudoexons aberrantly included in the cDNA of Patients #48 and #49 are presented as black boxes. The black arrows pointing to the right represent the cDNA START codons, the black lines pointing to the left represent the predicted STOP codon.

(p.Tyr109Profs*15). The significance of this cDNA change is unknown. It could be a byproduct of low-level aberrant splicing without any biological relevance or it might be used at some expression regulation level.

Patient 27 was investigated at the cDNA level, in order to analyze the effect of the homozygous mutation c.346 + 1G > T on the splice donor site of exon 2. The use of the mutated splice donor site (CAT/gt > CAT/tt) was replaced by the use of a potential splice donor site (TAG/gt) 23 bp upstream. This potential splice donor site is the one described above as being used in a small proportion of cDNA species in control cells. The c.346 + 1G > T mutation leads to the exclusive use of this potential splice donor site and the exclusion of the last 23 bp of exon 2 from the cDNA of the patient (r.324_346del) (Fig. 6). In addition, another splice change, leading to the exclusion of the 3′ part of exon 3 (r.379_589del), was also present on the same cDNA species (Fig. 6). This deletion results from the use of a potential splice donor site 211 bp upstream of the normal splice donor site of exon 3. Together, these two splice changes alter the unique cDNA species of Patient 27 so that in the resulting protein a stretch of 87 amino acids is replaced by a sequence of 9 other amino acids (Tyr109_Met195delins9). The frame is maintained till the usual *WISP3* termination codon, as the normal reading frame is restored in exon 4.

For Patient 48, we identified the c.156C > A mutation in mRNA obtained from cycloheximide treated cells, as expected from genomic sequencing. In addition, we identified a 131 bp insertion between exons 1 and 2, corresponding to a part of intron 1 (Fig. 6). A heterozygous base change (c.49-763G > T) located 14 bp upstream of the newly included genomic sequence was found and was likely to be responsible for the aberrant splicing of this region. This base change was not found in any of the 50 European control individuals tested. Also, compound heterozygosity for the c.156C > A and c.49-763G > T changes was confirmed by parental segregation. Aberrant splicing and inclusion of this genomic region in the cDNA leads to a frameshift and introduction of a premature stop codon resulting in a protein of 57 amino acids instead of 354 (p.Phe17Asnfs*42) if it escapes NMRD. The presence of this mutation in compound heterozygosity with the Cys52* mutation corroborated the clinical diagnosis of PPRD in this patient.

In Patient 49, the c.156C > A mutation identified by genomic sequencing was also detected in mRNA obtained from cycloheximide treated cells. In addition, a 27 bp insertion was detected in the cDNA at the end of exon 3, corresponding to the first 27 genomic bases of intron 3. At genomic sequencing, a heterozygous base change (c.589 + 27C > G) located 27 bp 3′ of the last nucleotide of exon 3 was identified, leading to the optimization of a potential splice donor site (from CACgt to CAGgt) 27 bp downstream of the usual one at the end of exon 3. This potential splice donor site had already been shown to be used in the case of a patient carrying a mutation at the usual exon 3 splice donor site (c.589G > C [Delague et al., 2005]). Thus, we hypothesize that the c.589 + 27C > G change causes a shift from the use of the normal splice site at the end of exon 3 to the use of the newly optimized splice site 27 bp further. This base change was not found in any of the 50 European control individuals tested. This splicing aberration leads to a frameshift and introduction of a premature stop codon resulting in a protein of 200 amino acids instead of 354 (p.Ala197Glyfs*5), if it escapes NMRD. This aberrant transcript would lack all domains except for the insulin-like growth factor-binding domain (Fig. 6). Again, compound heterozygosity for the c.156C > A and c.589 + 27C > G changes was confirmed by parental testing further supporting the diagnosis of PPRD in this patient.

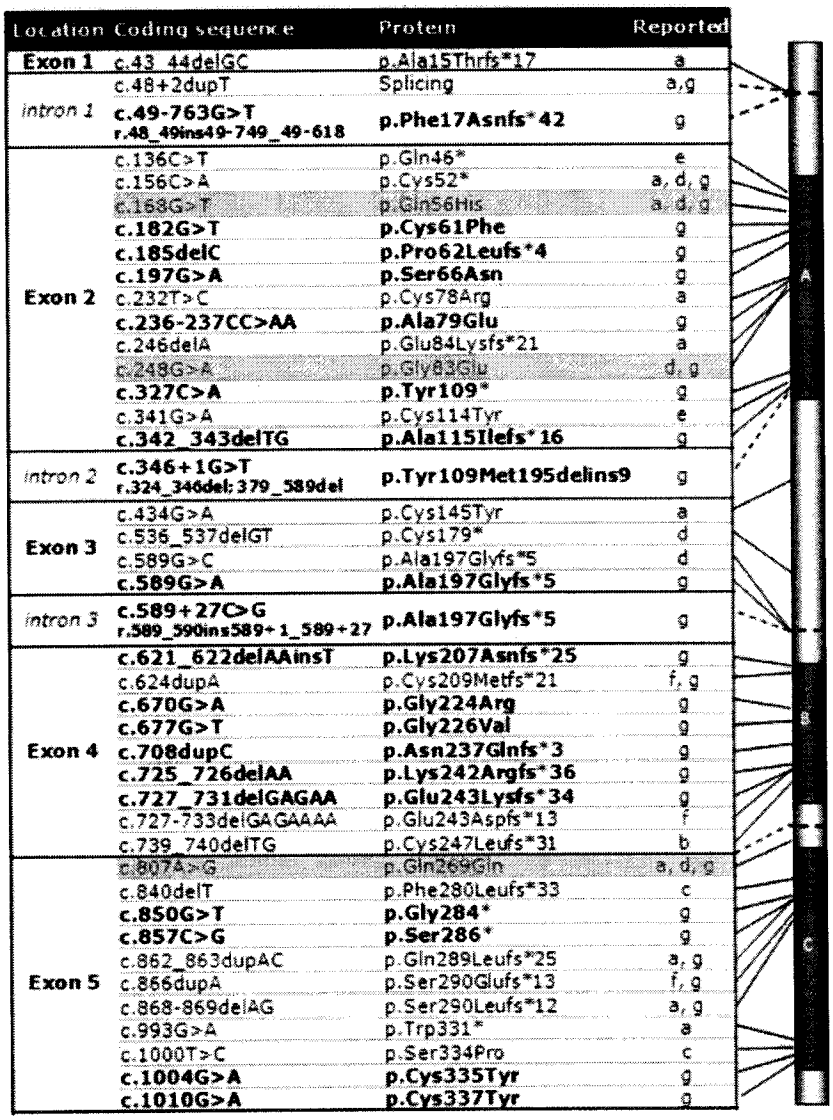
For Patient 50, no change was detected at the cDNA level as well as at genomic level. This patient was classified as phenotypically typical for PPRD because of the original description by Spranger et al. [1983b] (Patient 2, "B.K.") but the complete phenotype could not be reviewed as only pelvis radiographs were published (Fig. 4 in Spranger et al. [1983b]).

WISP3 Mutations and Polymorphisms in PPRD Patients

Of the 64 typical patients (from 49 unrelated families), 24 (18 families) were found to be homozygotes for the known and most common pathogenic mutation (c.156C > A; p.Cys52*), 25 (18 families) were homozygotes for other mutations, 12 (11 families) were compound heterozygotes for various mutations, and 2 were heterozygotes for the most common pathogenic mutation c.156C > A while no second mutation could be detected at the genomic level (Patients 48 and 49) (Table I). No mutation was found in Patient 50.

Altogether, we identified 20 previously unreported putatively pathogenic mutations (indicated in bold in Table I and Fig. 7), none of which were identified in over 100 control chromosomes. Parental segregation of mutations was confirmed in all 25 families for whom parental DNA was available. Figure 7 details all 39 *WISP3* mutations reported so far and their localization. Of all known WISP3 mutations, 22 are base pair substitutions, 11 are deletions (1–7 bp), 5 are duplications (1–2 bp), and 1 is a substitution/deletion (AA > T). Mutations are located throughout the *WISP3* gene in all the protein domains (see Fig. 7). Twenty-seven out of 39 known mutations lead to premature termination of the protein either because of a frameshift or because of a base substitution introducing a premature stop codon. For the rest, 11 mutations lead to amino acid substitutions and 1 leads to the removal of 87 and insertion of 9 new amino acids in the middle part of the protein with no premature termination (Patient 27).

The three previously reported *WISP3* polymorphisms were also identified in our cohort of patients. Of interest to note, all individuals carrying the c.156C > A (p.Cys52*) allele were also carriers of the c.248G > A



Location	Coding sequence	Protein	Reported
Exon 1	c.43_44delGC	p.Ala15Thrfs*17	a
	c.48+2dupT	Splicing	a,g
intron 1	**c.49-763G>T r.48_49ins49-749_49-618**	**p.Phe17Asnfs*42**	g
	c.136C>T	p.Gln46*	e
	c.156C>A	p.Cys52*	a, d, g
	c.168G>T	p.Gln56His	a, d, g
	c.182G>T	**p.Cys61Phe**	g
	c.185delC	**p.Pro62Leufs*4**	g
	c.197G>A	**p.Ser66Asn**	g
Exon 2	c.232T>C	p.Cys78Arg	a
	c.236-237CC>AA	**p.Ala79Glu**	g
	c.246delA	p.Glu84Lysfs*21	a
	c.248G>A	p.Gly83Glu	d, g
	c.327C>A	**p.Tyr109***	g
	c.341G>A	p.Cys114Tyr	e
	c.342_343delTG	**p.Ala115Ilefs*16**	g
intron 2	**c.346+1G>T r.324_346del;379_589del**	**p.Tyr109Met195delins9**	g
	c.434G>A	p.Cys145Tyr	a
Exon 3	c.536_537delGT	p.Cys179*	d
	c.589G>C	p.Ala197Glyfs*5	d
	c.589G>A	**p.Ala197Glyfs*5**	g
intron 3	**c.589+27C>G r.589_590ins589+1_589+27**	**p.Ala197Glyfs*5**	g
	c.621_622delAAinsT	**p.Lys207Asnfs*25**	g
	c.624dupA	p.Cys209Metfs*21	f, g
	c.670G>A	**p.Gly224Arg**	g
	c.677G>T	**p.Gly226Val**	g
Exon 4	**c.708dupC**	**p.Asn237Glnfs*3**	g
	c.725_726delAA	**p.Lys242Argfs*36**	g
	c.727_731delGAGAA	**p.Glu243Lysfs*34**	g
	c.727-733delGAGAAAA	p.Glu243Aspfs*13	f
	c.739_740delTG	p.Cys247Leufs*31	b
	c.807A>G	p.Gln269Gln	a, d, g
	c.840delT	p.Phe280Leufs*33	c
	c.850G>T	**p.Gly284***	g
	c.857C>G	**p.Ser286***	g
	c.862_863dupAC	p.Gln289Leufs*25	a, g
Exon 5	c.866dupA	p.Ser290Glufs*13	f, g
	c.868-869delAG	p.Ser290Leufs*12	a, g
	c.993G>A	p.Trp331*	a
	c.1000T>C	p.Ser334Pro	c
	c.1004G>A	**p.Cys335Tyr**	g
	c.1010G>A	**p.Cys337Tyr**	g

Figure 7. *WISP3* mutations and polymorphisms and corresponding position in the WISP3 protein domains. Previously unknown mutations reported in this article are indicated in bold. Polymorphisms are highlighted in gray. Changes were reported by: (a) Hurvitz et al. [1999]; (b) Ehl et al. [2004]; (c) Peng et al. [2004]; (d) Delague et al. [2005]; (e) Yue et al. [2009]; (f) Ye et al. [2012]; (g) the present paper. The WISP3 protein is depicted on the right, with the position of the mutations indicated with lines (dotted lines indicate an intronic position). Protein domains: (**A**) IGFBP (insulin-like growth factor-binding protein) N-terminal domain; (**B**) TSP (thrombospondin) type-1 domain; (**C**) CTCK (C-terminal cysteine knot-like) domain.

(p.Gly83Glu) polymorphism. Both changes are located in exon 2 and, as previously suggested [Delague et al., 2005], they are most likely the result of a founder effect.

DISCUSSION

WISP3 Mutational Spectrum

We report a series of 63 patients (from 48 families) with molecularly confirmed PPRD due to *WISP3* recessive mutations. The molecular spectrum is large, with mutations in all the domains of the WISP3 protein (Fig. 7). Pathogenicity of mutations appears clear from the predicted protein changes in the great majority of cases. Indeed, most of the mutations lead to premature stop codon with or without preceding frameshift, including the novel intronic mutations producing aberrant splicing. Of the eight novel missense mutations, four occur in cysteine residues; all these cysteines are highly conserved through species and through the several members of the CCN protein family and therefore these mutations are predicted to deeply affect the protein structure. The remaining four missense mutations have been found in compound heterozygosity with mutations predicting a premature stop codon and in patients with a typical PPRD phenotype and therefore are likely to be pathogenic.

Regarding splicing mutations, the previously reported c.48 + 2dupT (insT + 2IVS1 with the old nomenclature in Hurvitz et al. [1999]) and the novel c.346 + 1G > T (Patient 27) are easily detectable by sequencing of the coding exons and intron–exon boundaries, because of their proximity to the end of exon 1 and exon 2, respectively. We report, for the first time, the occurrence of intronic mutations affecting splicing in two patients in whom the clinical diagnosis was typical for PPRD but standard genomic sequencing failed to detect one pathogenic allele. Interestingly, these two mutations lead to aberrant splicing and inclusion of a pseudoexon in the *WISP3* cDNA, with predicted frameshift and premature stop codon (Fig. 6). We show that, even with low levels of expression of *WISP3* in cultured skin fibroblasts, these mutations are detectable and skin biopsy is therefore recommended in patients fulfilling the criteria for PPRD but with negative genomic molecular testing.

Most patients carried nonsense mutations in both *WISP3* alleles. We observed no phenotypic difference between patients homozygous for the recurrent Cys52* mutation and patients homozygous or compound heterozygous for other mutations predicting absence of the WISP3 protein. In addition, patients carrying a missense mutation in one of the two alleles did not show any significant difference in severity of phenotype compared to patients with two nonsense mutations in both alleles. Only two patients (Patients 20 and 36 in Table I) were homozygous for a missense mutation and their phenotype was not milder than that of patient with one or both alleles carrying a nonsense mutation. None of the heterozygotes, parents or sibs, had any joint related complaints.

PPRD Clinical Spectrum

Since the first report of *WISP3* mutations as causative for PPRD in 1999, only a few patients with confirmed molecular diagnosis of PPRD have been published. We report here our large series of patients and show that the clinical and radiological phenotype of patients with *WISP3* mutations is rather homogeneous with progressive worsening of symptoms and radiological changes during childhood and adolescence. Age of onset in all cases is between 3 and 6 years; in patients with earlier or later onset the diagnosis could not be confirmed. There is a remarkable delay in diagnosis from the time of onset of symptoms. This is certainly, at least partially, due to the lack of specific early signs but probably also due to a general low degree of awareness/suspicion of PPRD in differential diagnosis of other arthropathies. Turkish patients living in and out of Turkey seem to have a significantly shorter delay of diagnosis, perhaps because the presence of parental consanguinity suggests a genetic disorder to their physicians.

The first symptoms are usually gait anomalies, motor fatigability, and articular pain, mainly at the IPJ and knees. Early radiological signs are metaphyseal enlargement of IPJ (Fig. 2), often clinically mistaken as "joint swelling" (Fig. 1), short femoral neck with large femoral proximal epiphyses and reduced interarticular space at hips (Fig. 3). Stature is normal in infancy but the growth curve then typically deviates from the normal percentiles in childhood, with adult height being well below the 3rd percentile. Platyspondyly develops in late childhood and adolescence with characteristic anterior beaking of vertebral bodies at lateral spine radiographs leading to short trunk, dorsal kyphosis, and short stature; at early stages, spinal changes may be subtle (Fig. 4) and stature and proportions may still be normal. Typical posture in young adults is kyphotic, with flexum of elbows and knees and camptodactyly with knobbing of IPJ (Fig. 1). Scoliosis is less common being observed in only a few adult cases. Hip pain becomes the major problem in adolescence. Although longitudinal studies are lacking, hip replacement, performed already in the second decade of life, has proven successful in relieving of pain and restoring walking ability in several of the patients in our series. "Younger" patients (in their late teens or twenties) particularly appreciated being able to move independently. Although this information is anecdotal and possibly biased, all of the patients who underwent hip replacement were satisfied and some regretted having waited too long before accepting to undergo the procedure.

Pain is reported as an early symptom in only 15% of patients for whom clinical information was available. However, gait anomalies and fatigability were frequently reported as an early sign and may be a direct consequence of unrecognized pain in small children. IPJ "swelling" and stiffness are often early manifestations of PPRD, showing that hand involvement is one of the most typical and constant features of PPRD. The disorder is often misdiagnosed as seronegative JIA because initial clinical features mimic arthritis although autoimmunity markers and inflammatory parameters are usually normal [Spranger et al., 1983a], as confirmed in our series of patients. Affected subjects may receive ineffective anti-inflammatory and immune-suppressive treatments for several years before being diagnosed with PPRD [Ehl et al., 2004]. Intra-articular injections of corticosteroids lead to no or only transient relief of pain in a few documented cases. However, in adulthood, some patients showed phases with increased inflammatory parameters and some response to anti-inflammatory medications; a secondary inflammation following cartilage destruction might explain these findings. In several patients in their third and fourth decade, we observed periarticular calcifications at the elbow, knees, and hips, sometimes with osteophytic features (originating from bones) (Fig. 5), probably responsible for pain worsening and/or secondary inflammation. Recent cDNA microarray analysis in osteoarthritic cartilage has shown that *WISP3* is downregulated in osteophytic cartilage compared to articular cartilage [Gelse et al., 2012]. In PPRD, osteophytic calcifications appear much earlier than in non-genetic osteoarthritis; this could indicate that WISP3 is a key player in cartilage homeostasis

with its deficiency being sufficient to accelerate cartilage degeneration and osteophytic formation. As in other skeletal dysplasias, the radiological features become non-specific in adulthood and even the IPJ metaphyseal enlargement is often less evident.

WISP3-negative patients were referred because of variable degree of arthropathy associated with progressive stiffness in the absence of inflammation. However, either age of onset was outside of the typical postulated PPRD interval (3–8 years) or the contractures occurred without or with minimal pain. In some cases, peripheral joints were affected in a similar way as in PPRD but without spinal involvement. Differential diagnoses for these patients were, apart from JIA, other genetic non-inflammatory arthropathies as well as different forms of multiple epiphyseal dysplasia especially in early stages when the spinal and epiphyseal involvement may not be prominent. It is possible that some of these patients have mutations in other genes of the *Wnt* pathway accounting for a common pathophysiological mechanism responsible for clinical manifestations similar to PPRD.

PPRD is a progressive disorder with high burden of disease starting in childhood. Although its molecular basis was elucidated over 10 years ago, no progress has been made towards a specific treatment. The lack of phenotype in the knockout mouse has been one of the main limitations in studying the pathophysiology of the disease. Future research should be focused on the downstream effects of WISP3 on different extracellular matrix molecules in cartilage using appropriate in vitro models in order to identify possible therapeutic targets. In the meantime, improved recognition and earlier diagnosis may allow to assemble patient cohorts for prospective clinical studies. In spite of the nosologic entity being delineated almost 30 years ago, this study shows that the latency period between the onset of symptoms and the diagnostic confirmation remains very long. Increasing awareness of PPRD appears to be an essential first step to allow for earlier recognition.

ACKNOWLEDGMENTS

This work has been supported by the Swiss National Research Foundation, grant no. 310030_132940 to L.B. We thank Carole Chiesa, Valerie Pittet and Delphine Thuillard for technical assistance.

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